Cell-induced flow-focusing instability in gelatin methacrylate microdroplet generation

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Photo-crosslinkable gelatin methacrylate (GelMa) microspheres are applicable to deliver cells or drugs in biological or biomedical applications. To fabricate GelMa microdroplets, a flow focusing technique with advantages of size control and rapid production was used in a T-junction microfluidic device. Instability played an important role in promoting microdroplet uniformity. 5 wt. % GelMa prepolymer solution mixed with cells affected cell-induced instability. At low flow rate ratio of GelMa to mineral oil below 0.200, stability was maintained regardless of GelMa concentration (5 and 8 wt. %) and cell presence, which led to uniform microdroplet generation. In contrast, instability at high flow rate ratio above 0.200 was worsened by cell presence and unstable jetting length, resulting in the generation of non-uniform cell-laden microdroplets. Therefore, the effect of cell-induced instability on microdroplet generation was minimized at a low flow rate ratio. © *2014 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4880375]

I. INTRODUCTION

A variety of polymer microspheres have been developed as flexible platforms for biological or biomedical applications such as the controlled release of drugs and cell delivery.[1–8] Gelatin Methacrylate (GelMa) is one of the most favorable biopolymers for such applications that show a lengthy history of stability under numerous polymerization conditions on microscale. After the first introduction of GelMa by Van Den Bulcke *et al.* in 2000, various applications has been confirmed that GelMa is well suited in maintaining cell viability.[9] Nichol *et al.* reported that cell-laden GelMa microchannels are effective in creating perfusable engineered tissues.[10] Aubin *et al.* presented precise control of cell-laden GelMa patterned microgeometry for constructing 3D engineered tissues with specific elongation and alignment, *in vitro*.[11] Cha *et al.* covalently incorporated grapheme oxide into GelMa hydrogel for controlling mechanical properties of cell-laden hydrogels.[12] Recently, Shin *et al.* demonstrated GO-GelMa hybrid hydrogels for supporting cellular spreading and alignment with improved viability and proliferation in a 3D environment.[13]

Fabrication techniques such as solvent evaporation and spray drying have been used to generate polymer microspheres. Drug-loaded poly(l-lactide) (PLLA) microspheres and poly (l-lactide-co-glycolide) (PLGA) microspheres have been produced by solvent evaporation techniques, through microencapsulation by mechanical and chemical agitation, and by solvent evaporation in a core material.[14–16] PLGA microspheres for the encapsulation of darbepoetin alfa and quantum dot-encapsulated polymer microspheres were generated using a spray drying technique consisting of the dissolution of polymers, atomization, and solvent evaporation.[17–19] However, these conventional techniques are not suitable for controlling the size of cell-laden microspheres during their formation.

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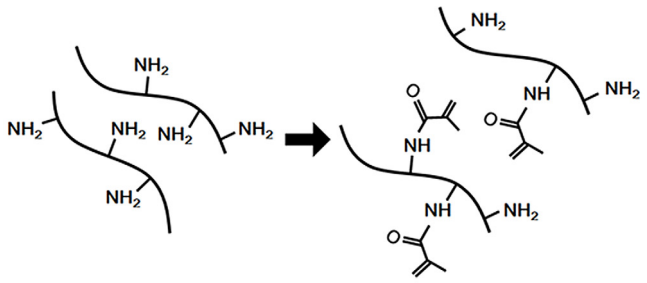
In this study, an emulsion technique incorporated with flow focusing in a T-junction channel was introduced for the rapid and uniform generation of microdroplets. They were fabricated using photo-crosslinkable GelMa with properties of biocompatibility and biodegradability. Instability as an important factor for stable microdroplet generation was investigated with regard to GelMa concentration and cell presence.

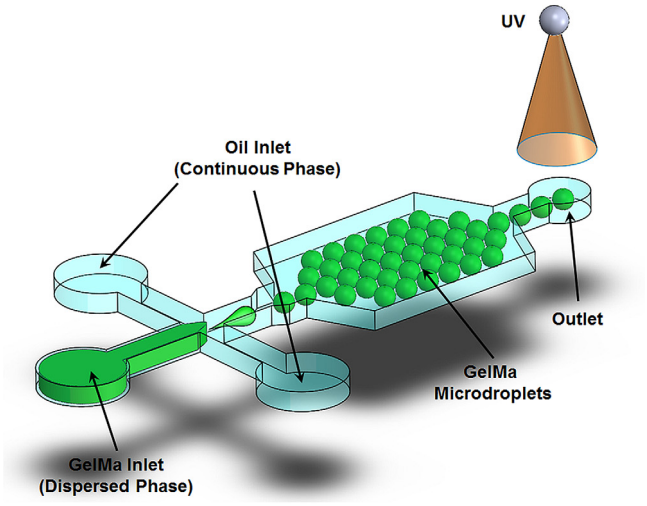
II. MATERIALS AND METHODS

A. Materials

Methacrylamide-modified gelatin (type A, bloom strength of 300, isolated from porcine skin; Sigma Aldrich, St. Louis, MO, USA) was synthesized by replacing the amine groups of the gelatin with methacrylamides (Fig. 1(a)). Both 5 g of gelatin (Sigma Aldrich) and 0.5 g of 4-(dimethlyamino)-pyridine (Sigma Aldrich) were dissolved into dimethyl sulfoxide (Sigma Aldrich) at 50 °C. During a continuous stirring of the solution at 50 °C, 2 ml of glycidyl methacrylate (Sigma Aldrich) was added into the solution at a rate of 0.5 ml/min. The reaction was allowed to proceed for 48 h in an N_2 gas environment. Then, using a dialysis membrane (molecular weight cut off 12 000–14 000 Da; Sigma Aldrich), the solution was purified with deionized water for seven days at 40 °C. A solid product of GelMa was obtained by lyophilization. In order to prepare 5 and 8 wt. % GelMa prepolymer solutions, the freeze-dried GelMa macromer was dissolved in phosphate buffered saline (pH 7.4) at 60 °C. Then, 0.2 wt. % of Irgacure 2959 (Ciba Specialty Chemicals, Basel, Switzerland) was mixed into the solution. Mineral oil was prepared with 20 wt. % Span 80 (Sigma Aldrich) as an emulsifier.



(a)



(b)

FIG. 1. (a) Methacrylamide-modified gelatin and (b) schematic of T-junction microdroplet generator (flow-focusing channel size: width and height = 100 μm).

B. Cell

NIH-3T3 mouse embryonic fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose; Invitrogen, Carlsbad, CA, USA). The DMEM was supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% fetal bovine serum (Invitrogen). The cells were incubated in a 5% CO_2 atmosphere at 37 °C. The media was changed every three days. The cells were prepared at a concentration of 10^7 cells/ml. The prepared NIH-3T3 cells were premixed with 5 wt. % GelMa prepolymer solution and infused into the microfluidic device for encapsulation. Here, a GelMa concentration of 5 wt. % was chosen to achieve the maximum number of cells encapsulated and the minimum density for photopolymerization.

The cell viability of the NIH-3T3 cells encapsulated in 5 wt. % GelMa microgels was investigated using Live/Dead Cell Viability Assay (Invitrogen) including calcein-AM and ethidium homodimer-1. The fluorescent markers in PBS (phosphate buffered saline) diluted to concentrations of 0.5 μl/ml and 2 μl/ml were mixed with microgels. They were incubated and rinsed using PBS. The live/dead cells were observed under an inverted fluorescent microscope at days 1 and 4.

C. Microdroplet generation in a microfluidic device

The SU-8 master with microchannels on a silicon wafer was prepared using standard photolithography. A mixture (10:1 ratio) of a silicone elastomer base and curing agent (Sylgard184 silicone elastomer kit; Dow Corning, Midland, Michigan, USA) was poured onto the master mold. Bubbles were removed in a vacuum chamber and then the mixture was cured at 80 °C for 1 h. The polydimethylsiloxane (PDMS) part was separated from the master. The inlets and outlets were punched out. The PDMS part was permanently bonded with a glass slide after 30 s of plasma treatment (Harrick Plasma, Ithaca, NY, USA) to fabricate the microfluidic device.

Fig. 1(b) describes the schematic procedure of GelMa microdroplet generation in a microfluidic device. The 5 and 8 wt. % GelMa prepolymer solutions (the dispersed phase) were perfused into the inlets at flow rates of 75, 100, 200, 300, and 400 μl/h, while mineral oil (the continuous phase) was injected into the device at a constant flow rate of 1000 μl/h. The flow rates of the fluids were precisely controlled using syringe pumps (KD Scientific Inc., Holliston, MA, USA).

The fabricated microfluidic device was placed on an inverted optical microscope (Olympus, Tokyo, Japan) to monitor microdroplet generation. Using the obtained images, both the droplet diameters and the jetting lengths were analyzed. The obtained data from ten measurements were expressed as means ± standard deviations. To determine the significance of representative experiments, statistical analysis using a one way ANOVA and student's t-test was performed. P values less than 0.05 were considered to be statistically significant.

D. Photopolymerization

Microdroplets flowing out from the outlet were exposed to a UV light source (OmniCure S2000, Lumen Dynamics, ON, Canada). The UV source was placed at a distance of 8 cm above the microdroplets with curing parameters of 800 mW (power), 17.5 mW/cm^2 (intensity), and 30 s (exposure time) to achieve photo-polymerization of 5 wt. % GelMa microdroplets in mineral oil.

III. RESULTS AND DISCUSSION

The T-junction microfluidic device using PDMS flow-focusing channels rapidly generated aqueous GelMa microdroplets with sizes controllable according to the changes in flow rates. In a flow-focusing geometry, the surfactant (20 wt. % Span 80) added into the mineral oil phase played two crucial roles; first, it created enough shear stress to squeeze and break the dispersed phase (GelMa) into microdroplets, and second, it stabilized the formed microdroplets into spherical shapes. Different droplet sizes and jetting lengths as a function of flow rate were observed in accordance with the GelMa concentrations (5 and 8 wt. %) and the presence of

cells, as shown in Fig. 2. The overall trend was that the diameters of the microdroplets increased and the jetting shapes became longer and thicker with the increase in GelMa flow rates. At a fixed flow rate of the oil phase (1000 μl/h), the shear stress acting on the dispersed phase was reduced when the flow rate of the GelMa prepolymer solution increased. Thus, the droplet size and jetting length became larger and longer, respectively.

At low flow rates of the GelMa prepolymer solution, below 200 μl/h, uniform-sized microdroplets were produced regardless of GelMa concentration and cell presence. However, at high flow rates above 200 μl/h, an unstable jetting regime was observed when the cells were present in the dispersed phase, while a stable jetting regime was still maintained in the absence of cells.

Fig. 3 indicates the size differences in 5 wt. % GelMa microdroplets (with and without cells) and 8 wt. % GelMa microdroplets (without cells) at different flow rate ratios (ranging from 0.075 to 0.400 between the dispersed phase and the continuous phase). In general, as the flow rate ratio increased, the diameter of the GelMa microdroplets proportionally increased. At low flow rate ratios between 0.075 and 0.100, all microdroplets followed the same inclination in the growth of droplet diameter, from 32.7 ± 1.0 to 42.9 ± 1.4 μm, regardless of GelMa concentration and cell presence. However, at high flow rate ratios from 0.200 to 0.400, there was a different tendency in the diameters of the microdroplets, depending on GelMa concentration and cell presence. As the flow rate of GelMa prepolymer solution increased, the effect of viscosity of GelMa became dominant. For example, at a flow rate ratio of 0.200, 8 wt. % GelMa microdroplets without cells showed the greatest increase in diameter, up to 71.4 ± 3.1 μm, while the diameters of 5 wt. % GelMa microdroplets without cells increased only up to 54.3 ± 2.8 μm. The diameters of 5 wt. % GelMa microdroplets with cells were located between the diameters of 5 and 8 wt. % GelMa microdroplets without cells, showing the largest variation in diameter due to worsened stability. Interestingly, above a flow rate ratio of 0.200, the presence of cells

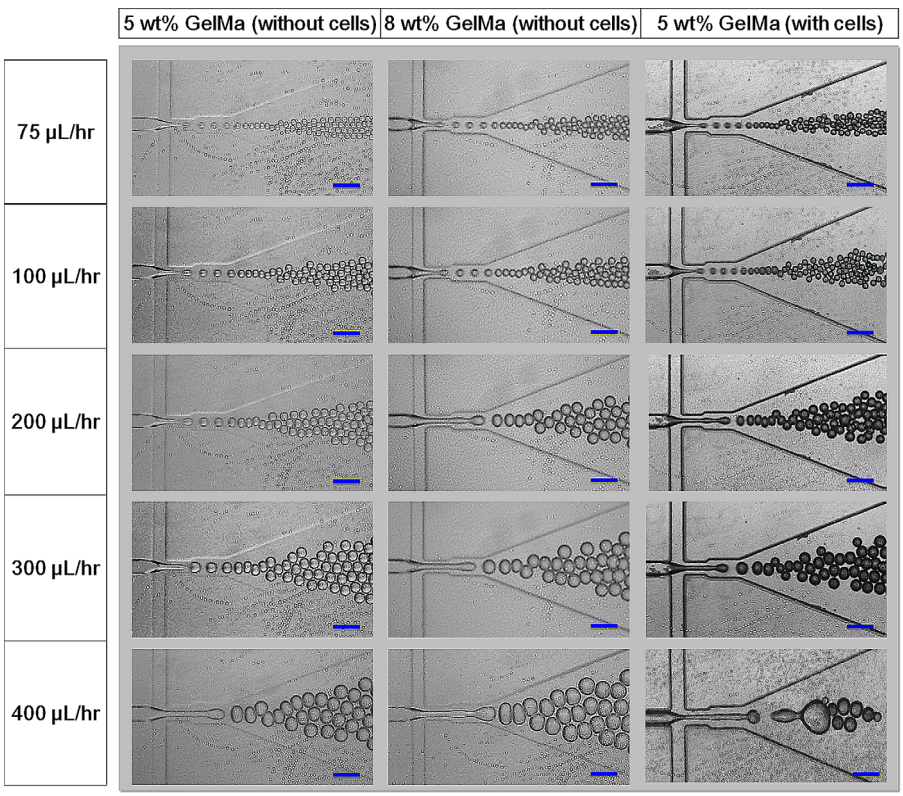


FIG. 2. Effect of GelMa concentration (5 and 8 wt. %) and cell presence on microdroplet generation with changing flow rates of GelMa (75 to 400 μl/h). Scale bar indicates 200 μm. (Cell concentration = 10^7 cells/ml.)

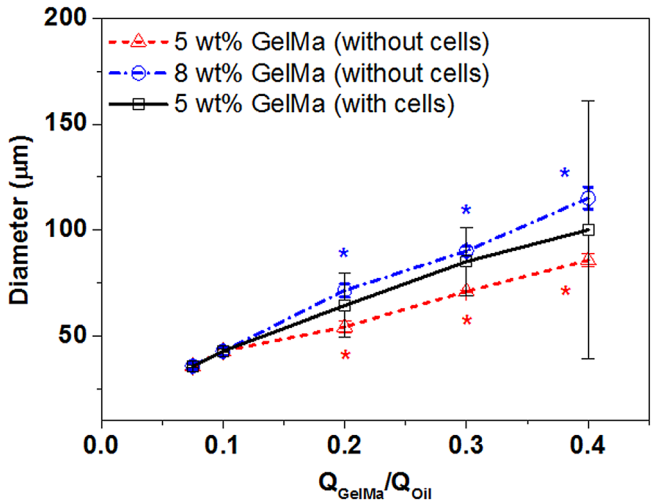


FIG. 3. A size comparison of the GelMa microdroplets according to GelMa concentration (5 and 8 wt. %) and cell presence as a function of flow rate ratio. (Q_{GelMa}/Q_{oil} = flow rate of GelMa/flow rate of oil.) (* $P < 0.05$, error bars represent the standard deviations.)

in 5 wt. % GelMa prepolymer solution drastically increased instability in a flow-focusing channel, thus resulting in the increase of variations in the microdroplet generation. At a flow rate ratio of 0.400, the extremely wide diameter range of 39.2 to 161.0 μm was observed in the cell-laden microdroplet generation.

The aforementioned high variation in diameters seemed to be caused by the sharp fluctuations in jetting length. As seen in Fig. 4, overall, with the increase of flow rate ratio, the jetting length became longer in the order of 5 wt. % GelMa without cells, 5 wt. % GelMa with cells, and 8 wt. % GelMa without cells. However, at a flow rate ratio of 0.400, 5 wt. % GelMa microdroplets with cells showed the largest jetting length (612.9 ± 109.8 μm) due to cell-induced instability. When the viscosity of the solution was increased due to the presence of cells, the breakup of microdroplets from the GelMa prepolymer solution was inconsistent, resulting in the fluctuations in the jetting shape. Thus, the instability was worsened markedly, creating non-uniformity in the microdroplet generation. As a result, when producing cell-laden microdroplets for biomedical applications requiring large numbers of cells, a low flow rate ratio is required so that the effect of cell-induced instability becomes minimized.

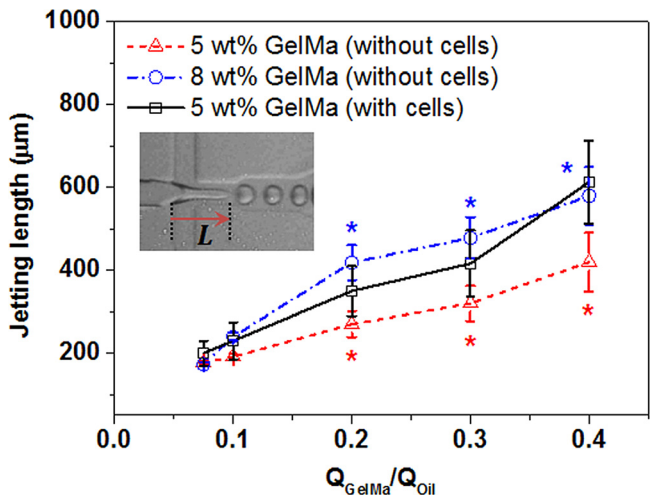


FIG. 4. The effect of GelMa concentration (5 and 8 wt. %) and cell presence on the jetting length (L) as a function of flow rate ratio. (* $P < 0.05$, error bars represent the standard deviations.)

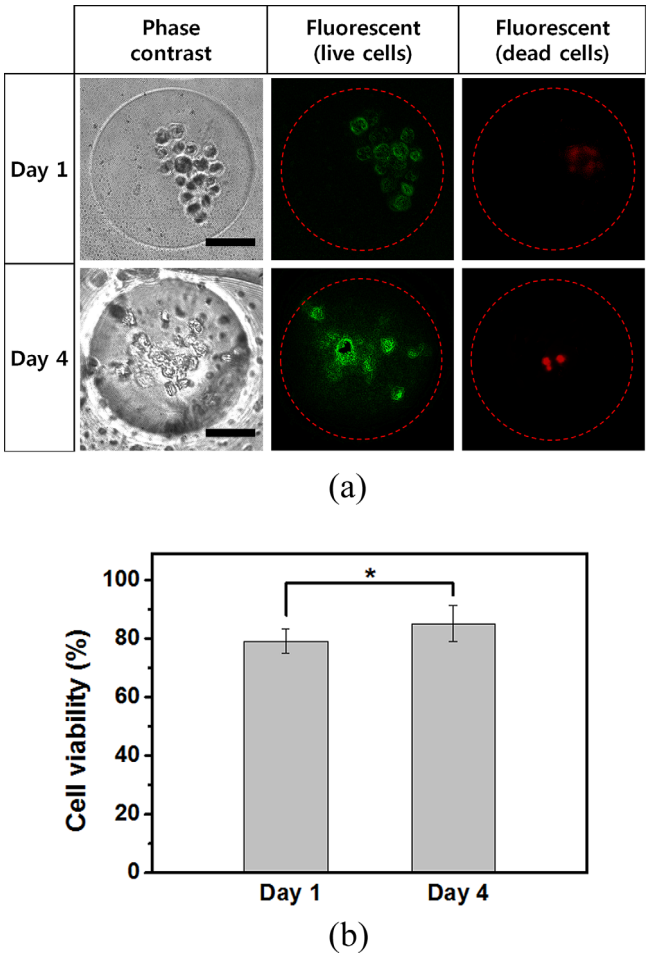


FIG. 5. The viability of NIH-3T3 cells encapsulated in 5 wt. % GelMa microgels. (a) Phase contrast and fluorescent images of live and dead NIH-3T3 cells at days 1 and 4. (Scale bar = 30 μm). (b) Cell viability at days 1 and 4. (* $P < 0.05$, error bars represent the standard deviations.)

The viability of NIH-3T3 cells encapsulated in 5 wt. % GelMa microgels was investigated, as shown in Fig. 5. The phase contrast and fluorescent images on the viability and morphology at days 1 and 4 were shown in Fig. 5(a). The Initial spherical shape of cells was observed at day 1, and cell proliferation was noted at day 4. In Fig. 5(b), Cell viability of $79.2 \pm 4.2\%$ was measured at day 1, and the value increased at day 4 $85.2 \pm 6.2\%$, confirming the stability of cell viability in GelMa microspheres.

IV. CONCLUSIONS

Using T-junction microdroplet generator, the instability of the microdroplet generation was investigated depending on GelMa concentration and cell presence. At low flow rate ratio below 0.200, the enhancement of stability in droplet generation was achieved regardless of GelMa concentration and cell presence. As the viscosity of GelMa prepolymer solution became dominant above the flow rate ratio of 0.200, cell-induced instability caused non-uniform droplet generation with high variance in diameter. These results suggest the encapsulation of a large number of cells at low flow rate ratio for biomedical applications.

ACKNOWLEDGMENTS

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